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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/772,416	02/06/2004	Jerzy Zawistowski	Shelf Life US	2056

7590 04/30/2007

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EXAMINER	
PADEN, CAROLYN A	

ART UNIT	PAPER NUMBER
1761	

MAIL DATE	DELIVERY MODE
04/30/2007	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	10/772,416	ZAWISTOWSKI, JERZY	
	Examiner	Art Unit	
	Carolyn A. Paden	1761	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *31 August 2005*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-37* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1-37* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

Claims 1-5, 10-21, 23-26, 30-37 are rejected under 35 U.S.C. 103(a) as being unpatentable over Wang (2002) and Yoshida (2003) taken together.

Each of Yoshida and Wang discloses the antioxidant activity of phytosterols and phytosterols conjugates in vegetable oils. Yoshida discloses that phytosterols acts to protect and stabilize cell membranes from oxidation at page 279, near bottom of column 1. The claims appear to differ from Wang and Yoshida in the recitation of the antimicrobial activity of phytosterols. But the antimicrobial effect of phytosterols is an inherent property of the compound. Further one of ordinary skill in the art would expect phytosterols to protect foods from microorganisms, fungus, viruses or yeast by stabilizing the cell membranes of the food. Also phytosterols would also be expected to protect foods from microorganisms by slowing the breakdown of fats to more digestible and lower molecular weight chemical components.

Claims 6, 7, 9, 22, 27, 28 and 35 are rejected under 35 U.S.C. 103(a) as being unpatentable over Wang and Yoshida taken together as applied to claims 1-5, 10-21, 23-26 and 30-37 above, and further in view of Hallstrom.

The claims appear to differ from Wang and Yoshida in the recitation of the extent of free sterols in the composition. Hallstrom teaches a composition containing about 15% free sterol and hard butter. With the disclosure of Wang and Yoshida in hand, it would have been obvious to one of ordinary skill in the art to expect the fat of example 1 of Hallstrom to be preserved by the phytosterols of the composition.

Claims 8 and 29 are rejected under 35 U.S.C. 103(a) as being unpatentable over Wang and Yoshida taken together as applied to claims 1-5, 10-21, 23-26 and 30-37 above, and further in view of van Amerongen (6,492,538).

The claims appear to differ from Wang and Yoshida in the recitation that the composition contains a combination of free and esterified phytosterols in it. Van Amerongen teaches hydrolyzing phytosterols to obtain a composition containing 50% phytosterols esters (column 4, lines 4-11). It would have been obvious to one of ordinary skill in the art to expect

the spread of van Amerongen to be stabilized by the phytosterols in the composition.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Carolyn A Paden whose telephone number is (571) 272-1403. The examiner can normally be reached on Monday to Friday from 7 am to 3:30 pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Milton Cano, can be reached on (571) 272-1398 or by dialing 571-272-1700. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Carolyn Paden

CAROLYN PADEN 7.24.07
PRIMARY EXAMINER

Notice of References Cited	Application/Control No. 10/772,416	Applicant(s)/Patent Under Reexamination ZAWISTOWSKI, JERZY	
	Examiner Carolyn A. Paden	Art Unit 1761	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-5,547,677	08-1996	Wright, D. Craig	424/401
*	B	US-6,419,963	07-2002	Niazi, Sarfaraz K	424/757
*	C	US-4,160,850	07-1979	Hallstrom et al.	426/601
*	D	US-6,492,538	12-2002	van Amerongen et al.	554/229
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FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
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NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Gordon, M. H. 1983. Food Chemistry 10:141-147.
	V	Wang, T. 2002. JAOCS 79(12)1201-1206.
	W	Yoshida, Y. 2003. J. Nutr. Sci. Vitaminol. 49:277-28[illegible]
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Carolyn Paden 4.27.07

Food Chemistry 10 (1983) 141–147

The Effect of Sterols on the Oxidation of Edible Oils

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Department of Food Science, Queen Elizabeth College, Campden Hill Road, London W8 7AH, Great Britain

(Received: 29 January, 1982)

ABSTRACT

The effect of sterols on the oxidation of a triglyceride mixture, similar in composition to olive oil, has been studied at 180°C. Δ^5-Avenasterol and fucosterol are effective as antioxidants whilst other sterols, including cholesterol and stigmasterol, are ineffective. The antioxidant effect of Δ^5-avenasterol increases with concentration in the range 0·01% to 0·1%.

An hypothesis is presented to explain the effectiveness of the sterols as antioxidants. It is concluded that lipid free radicals react rapidly with sterols at unhindered allylic carbon atoms. Isomerisation leads to a relatively stable allylic tertiary free radical, which is slow to react further, and this interrupts the autoxidation chain.

INTRODUCTION

Vegetable oils containing polyunsaturated fatty acids oxidise rapidly at elevated temperatures. This results in a reduction in the essential fatty acid content and darkening and polymerisation of the oil. Common antioxidants, including butylated hydroxyanisole (BHA) or tocopherols, retard oxidation at ambient temperatures but they are ineffective at retarding oxidation at the high temperatures (180 °C) required for frying food (Ramel *et al.*, 1965). Methyl polysiloxanes are added to vegetable oils to retard oxidation in heated oils (Martin, 1953). It has also been

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Food Chemistry 0308-8146/83/0010-0141/$03·00

claimed that triterpene alcohols and hydrocarbons (Boskou & Katsikas, 1979), or certain sterols (Sims *et al.*, 1972; Boskou & Morton, 1976) retard the deterioration of oils during prolonged heating. It is believed that an ethylidene group in the side chain of the sterol is required for this antioxidant effect (Sims *et al.*, 1972).

This work is directed towards gaining a better understanding of the effect of sterol structure and concentration on the rate of oxidation of a vegetable oil. The effect of added sterols on the drop in iodine value of a model triglyceride mixture has been studied during oxidation at 180 °C. The triglyceride mixture studied was similar in fatty acid composition to olive oil, and was free of unsaponifiable matter, which might exhibit synergistic effects with added sterols. Changes in iodine value, which accompany the oxidation of oils at elevated temperatures (Waltking & Zmachinski, 1970), have been confirmed by refractive index and fatty acid determinations.

MATERIALS AND METHODS

Materials

BHA, cholesterol and trioleylglycerol, prepared from technical oleic acid, were purchased from BDH Chemicals Ltd. The fatty acid composition of the triglyceride mixture was found to be 76% oleic acid, 8% saturated fatty acids (stearic, palmitic, myristic), 7% palmitoleic acid, 6% linoleic acid with traces of other fatty acids. Stigmasterol and α-tocopherol were purchased from the Sigma Chemical Company.

Fucosterol and Δ^5-avenasterol were isolated in the laboratory from brown algae (*Fucus vesiculosus*) (Heilbron *et al.*, 1934) and green algae (*Ulva lactuca*) (Gibbons *et al.*, 1968), respectively. The electron impact mass spectra of the sterols were in agreement with reported spectra (Bergman *et al.*, 1965; Gibbons *et al.*, 1968). The sterols appeared pure by GLC under the conditions commonly used for analysing sterol mixtures (Boskou & Morton, 1975).

Methods

Samples of the triglyceride mixture (technical trioleylglycerol) (50 g) containing additives were heated at 180 ± 5 °C for 8 h a day with cooling

to room temperature at night. Aliquots (5 g) were removed after 24 h, 48 h, 72 h total heating time and the samples were stored under nitrogen in a refrigerator until required for analysis. A sample of trioleylglycerol without additives was heated as a control in each experiment.

Iodine values are the mean of duplicate determinations by Wij's method, according to the British Standards specification. Gas chromatographic analysis was performed on methyl esters prepared according to Brockerhoff (1965). A 2 m column of DEGS (10% on Chromosorb W, 80–100 mesh) was used at 180 °C for the analysis. Antioxidant activity was calculated in terms of the protective index (PI) used by Sims *et al.*, 1972:

$$PI = \frac{\text{Change in iodine value of a control sample}}{\text{Change in iodine value of a sample containing additives}}$$

PI > 1 indicates that the additive has an antioxidant effect.

RESULTS AND DISCUSSION

Δ^5-Avenasterol and fucosterol are both effective at minimising the fall in iodine value during heating at 180 °C (Fig. 1). Stigmasterol, cholesterol,

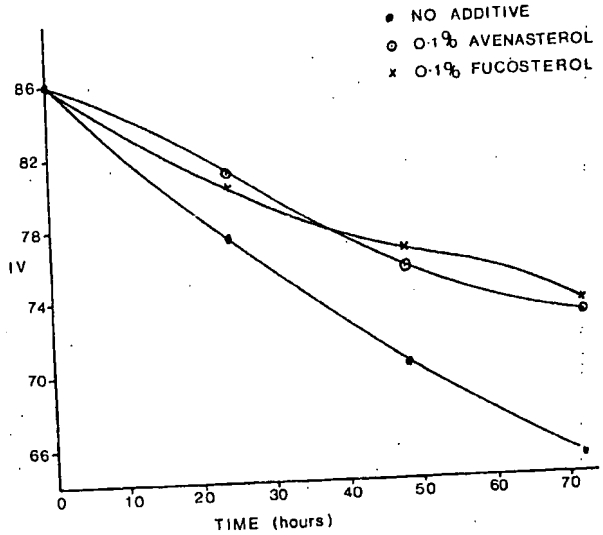


Fig. 1. The Iodine Value (IV) of trioleylglycerol during heating at 180 °C in the presence or absence of additives.

TABLE 1
Protective Indices (PI) of Additives During the Heating of Technical Trioleylglycerol at 180 °C

Additive	*Concentration* (%)	*PI after heating for:* 24 h	48 h	72 h
α-Tocopherol	0·02	0·97	1·01	1·01
BHA	0·02	0·96	1·04	1·00
Cholesterol	0·1	0·96	1·12	1·12
Stigmasterol	0·1	0·95	1·11	1·09
Fucosterol	0·1	1·52	1·71	1·66
Δ^5-Avenasterol	0·1	1·78	1·69	1·59
Δ^5-Avenasterol	0·05	1·16	1·19	1·09
Δ^5-Avenasterol	0·01	1·10	1·09	1·07

BHA and α-tocopherol are relatively ineffective as antioxidants, having PI values $<1·2$, whilst Δ^5-avenasterol and fucosterol have PI values $>1·5$, when present at a concentration of 0·1 % (Table 1). The magnitude of the antioxidant properties of Δ^5-avenasterol can be appreciated when one considers the effect on the essential fatty acid content of the oil (Fig. 2). The time at 180 °C for a 50 % reduction in linoleic acid content in the

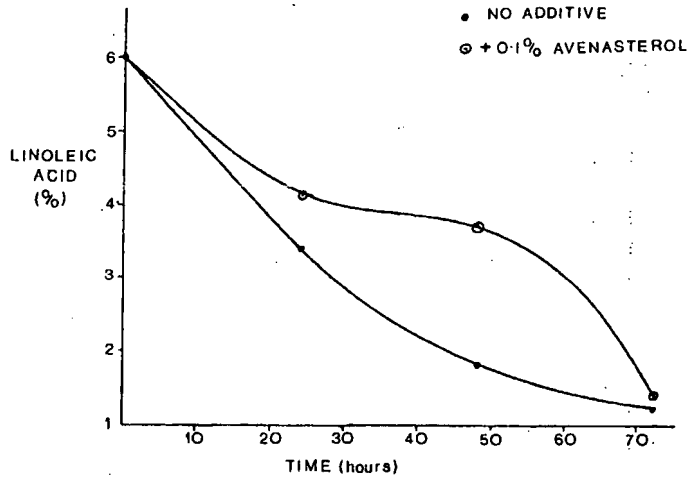
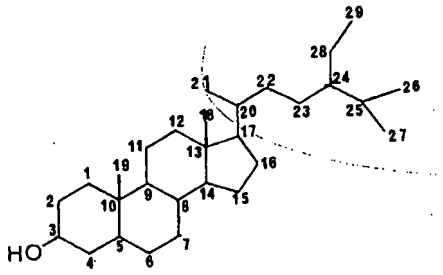


Fig. 2. The linoleic acid content of trioleylglycerol during heating at 180 °C in the presence or absence of Δ^5-avenasterol.

absence of sterols is approximately 28 h whilst, in the presence of 0·1 % Δ^5-avenasterol, the time increases to approximately 59 h.

The effectiveness of Δ^5-avenasterol as an antioxidant increases with concentration in the range 0·01–0·1 %. Δ^5-Avenasterol is present in olive oil at <0·02 % (Itoh *et al.*, 1973) and it is clear that the sterol has a very slight antioxidant effect at this concentration in oil heated at 180 °C. However, synergistic effects with other unsaponifiable components may occur in olive oil.

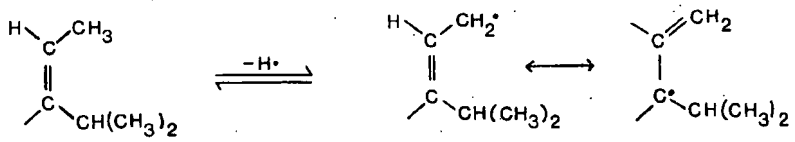
Previous work (Sims *et al.*, 1972) has shown that the order of effectiveness as antioxidants in safflower oil at 180 °C is vernosterol > Δ^7-avenasterol > fucosterol (Fig. 3) whilst spinasterol, ergosterol, lanosterol, β-sitosterol, stigmasterol and cholesterol have no significant



STEROL	DOUBLE BONDS
Vernosterol	8,14,24(28)
Δ^5-Avenasterol	5,24(28)
Fucosterol	5,24(28)
Stigmasterol	5,22
Δ^7-Avenasterol	7,24(28)

Fig. 3. The structure of plant sterols.

antioxidant activity. The 4-methylsterol, citrostadienol, is also effective as an antioxidant. These observations can be explained by the hypothesis that sterols with a structure that allows them to react rapidly with lipid free radicals to form relatively stable free radicals are effective as antioxidants. Relatively stable free radicals interrupt the triglyceride autoxidation chain reaction. The antioxidant effect is greatest when free radical formation from the sterol is relatively rapid due to the presence of unhindered hydrogen atoms on an allylic carbon atom, and when the free radical thus formed can isomerise to a tertiary free radical (as shown below), which is known to be relatively stable (Nonhebel & Walton, 1974).



Thus, sterols with an ethylidene group in the side chain are most effective as antioxidants, but a further antioxidant effect may arise from the presence and position of one or more endocyclic double bonds. Vernosterol has an antioxidant effect arising from the rapid formation of free radicals at C-29, but slower formation of free radicals at C-11 or C-16, which are stabilised by delocalisation over two double bonds, also contributes to the antioxidant activity of the sterol. Thus, vernosterol is more effective than sterols with one endocyclic double bond; for example, fucosterol.

Stigmasterol does not have significant antioxidant activity, despite being able to form tertiary free radicals at the allylic carbon atoms C-20 and C-24. Presumably the rate of loss of hydrogen atoms at tertiary carbon atoms is slow because of steric hindrance to the approach of a free radical.

Future work is intended to investigate whether differences exist in the antioxidant effects of Δ^5 and Δ^7 sterols, and also to study whether synergistic effects occur in the antioxidant activity of sterols.

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Antioxidant Activity of Phytosterols, Oryzanol, and Other Phytosterol Conjugates

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[a]Department of Food Science and Human Nutrition, Iowa State University, Ames, IA 50011, and [b]Eastern Regional Research Center, ARS, USDA, Wyndmoor, Pennsylvania 19038

ABSTRACT: Antioxidant activity of phytosterols, oryzanol, ferulic acid ester of sterols, corn fiber oil, and rice bran oil was investigated. Commercial soybean oil and distilled soybean oil FAME were used as substrates for both oxidative stability determination and viscosity analysis after the oil was oxidized. At low concentration, these materials did not improve the oxidative stability of the oil substrates, although the viscosity tended to be reduced slightly. The antipolymerization activity of steryl ferulate was higher at higher concentration than at lower concentration, and steryl ferulate was more effective than oryzanol. Rice bran oil showed very good antioxidant and antipolymerization activities.

Paper no. J10247 in *JAOCS 79*, 1201–1206 (December 2002).

KEY WORDS: Antioxidant, antipolymerization, corn fiber oil, ferulic acid ester of phytosterol, phytosterols, rice bran oil.

Phytosterols contained in vegetable oils are hypocholesterolemic (1–3). These phytochemicals and their derivatives may also be potent antioxidants (4–7). The avenasterol of rice bran oil acts as an antioxidant at elevated or frying temperatures owing to the ethylidene group on the side chain of the molecule. Its antioxidant activity has been attributed to the formation of an allylic free radical and its isomerization to other relatively stable free radicals (7). Oryzanol, a group of ferulic acid esters of triterpene alcohols present in rice bran oil, also has been shown to possess strong stabilization effects during frying applications (6). The antioxidant effects of ferulates can similarly be explained by the formation of several resonance-stabilized structures.

Corn fiber oil is an unusual oil in that it contains a very high concentration of total sterols, up to ~14% of the crude oil, compared with less than 1% in most refined vegetable oils (8–12), and it has been shown to lower serum cholesterol levels in several animal models (11,12). In addition to free phytosterols and phytosterol fatty acyl esters, which have been granted a rare health claim by the U.S. Food and Drug Administration for lowering both LDL-cholesterol levels and the risk of heart disease, corn fiber oil also contains high levels of sitostanyl-ferulate derivatives. These sitostanyl-ferulates also were very effective in lowering cholesterol in an animal model in a preliminary study (13). If these steryl ferulate derivatives are both biologically active and chemically suitable as antioxidants at high temperature, a multifunctional food ingredient or nutraceutical could be developed. The objective of this research was to investigate the antioxidant and antipolymerization activities of phytosterols/phytostanols and their ferulic acid esters.

EXPERIMENTAL PROCEDURES

Antioxidant activity of various natural and synthetic agents. Sitostanyl ferulate was synthesized (14) at a purity of >99%. Sitostanol was obtained from Research Plus (South Plainfield, NJ). Soy sterols, obtained from Archer Daniels Midland (Decatur, IL), is a mixture of naturally occurring free sterols, consisting of β-sitosterol (45.7%), campesterol (27.3%), stigmasterol (15.3%), and brassicasterol (4.4%). It has a purity of 95%. γ-Oryzanol was obtained from CTC Organics (Atlanta, GA). Crude corn fiber oil was extracted with hexane from corn fiber (obtained *via* conventional wet-milling, air-drying, and grinding to 20 mesh), and it contained 5.9% FA ester of sterols, 2.1% free sterols, 5.4% ferulic acid ester of sterols (9), and low levels (<1%) of tocopherols, since the fiber was not heated prior to extraction (15). Crude rice bran oil, obtained from Riceland Foods (Stuttgart, AR), contained about 1.86% oryzanols and 0.3% avenasterol (6). Ferulic acid, gallic acid, TBHQ, and δ-tocopherol were purchased from Sigma Chemical (St. Louis, MO). δ-Tocopherol was chosen as one of the controls because it is generally considered as the most active isomer among the other tocopherols.

Two substrates—commercially refined, bleached, and deodorized (RBD) soybean oil and distilled soybean oil methyl esters (FAME)—were used to test the antioxidant activity of these phytosterol compounds. A short-path molecular still (Pope Scientific, Saukville, WI) was used to distill FAME in order to obtain tocopherol-free substrate. Two concentrations of various compounds, one at 6 μmol/5 g substrate and another at 12 μmol/5 g, were tested. The concentration of 6 μmol/5 g was equivalent to the regulated application of TBHQ, which is 0.02% of the oil. The concentrations of antioxidants contained in corn fiber oil and rice bran oil were prepared by adding the calculated amount of oils that would

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give thecorrect sterol concentrations. The average M.W. of the three major oryzanols (in γ-oryzanol) was estimated to be 580. Oxidative stability index (OSI) was measured according to AOCS standard method Cd 12b-92 (16) with the Omnion OSI instrument (Omnion, Rockland, MA). Viscosity of oil or FAME was measured using a Brookfield viscometer (Stoughton, MA) after the reaction had reached the OSI end point.

Antioxidant activity of selected compounds at various concentration levels. Commercial RBD soybean oil was used as substrate to test antioxidant and antipolymerization activities of the selected compounds at various concentration levels. Four concentrations, i.e., 12, 24, 36, and 60 μmol/5 g substrate, were used to test the antioxidant activity of sitostanyl ferulate, oryzanol, soybean sterols, rice bran oil, and ferulic acid. The reason for using various concentrations of an antioxidant is that, although a positive dose–response relationship usually is present, such relationships may be different for different antioxidants. Examining the dose–response relationship is important so the optimal antioxidant concentration can be determined.

Statistical analysis. The general linear model of the SAS program (17) was used for the ANOVA. To examine the effect of type of compound and concentration on antioxidant activity, a two-factor factorial design was used, with the type and concentration as two factors. Least significant differences (LSD) were calculated (P = 0.05) to compare treatment means.

RESULTS AND DISCUSSION

Antioxidant activity of various natural and synthetic compounds. The values of OSI and viscosity of oils and FAME with various compounds at two concentration levels are shown in Table 1. The results of statistical analysis are shown in Table 2.

Different compounds had significantly different OSI values in both FAME and oil substrates. FAME were oxidized much faster than oil because the natural antioxidant was removed by distillation purification of the FAME. Compared with blank FAME that did not contain any added chemical, the oxidative stability of FAME was significantly improved by adding TBHQ and δ-tocopherol. Rice bran oil improved FAME stability considerably as well. Oil stability was improved by adding gallic acid, TBHQ, and rice bran oil, but δ-tocopherol did not influence stability as much as expected. Various sterols and the sterol-rich corn fiber oil did not seem to improve stability. In addition, higher concentration of the sterols did not significantly improve the stability of either system.

The effect of various compounds on polymerization reaction and viscosity development in both FAME and oil substrates was significant. TBHQ and δ-tocopherol were the most effective compounds in preventing polymerization of the FAME. Although gallic acid was quite effective in improving OSI, it did not prevent viscosity development to a similar degree. Various sterols generally lowered viscosity slightly, particularly at the higher concentration (12 μmol/5 g).

TABLE 1
Antioxidant Activity of Various Natural and Synthetic Agents Tested in Distilled FAME and Commercial Soybean Oil[a]

		OSI (h)				Viscosity (cP)			
	Conc.	FAME		Soybean oil		FAME		Soybean oil	
	(μmol/5 g)	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Sitostanol-ferulate	6	5.48	0.60	11.30	0.35	29.60	2.97	166.70	21.92
	12	6.28	1.10	11.70	0.07	25.15	0.49	508.00	12.73
Sitostanol	6	4.80	0.35	11.53	1.38	36.45	7.28	293.45	46.46
	12	4.90	0.71	11.05	0.42	28.55	1.63	957.90	154.29
Soy sterols	6	5.08	1.03	12.43	0.53	33.90	4.10	190.10	12.02
	12	4.80	0.92	11.28	0.46	30.30	2.97	808.20	105.50
Oryzanol	6	6.05	0.21	12.18	0.32	28.65	1.63	217.95	31.04
	12	6.28	0.60	11.53	0.11	27.45	0.21	875.30	0.71
Corn fiber oil	6	5.23	0.39	10.75	0.57	32.95	1.20	323.90	22.06
	12	5.45	1.20	10.30	0.42	33.45	2.76	1538.50	440.53
Rice bran oil	6	9.18	0.39	13.78	0.04	29.90	5.09	136.85	39.81
	12	11.95	0.64	14.68	1.03	20.10	3.82	521.25	29.49
δ-Tocopherol	6	12.40	2.62	12.25	0.42	13.65	6.15	196.55	55.65
	12	17.78	0.95	12.55	0.35	7.69	0.81	638.20	2.40
Ferulic acid	6	6.60	1.13	12.55	0.42	28.45	2.33	169.40	39.32
	12	7.05	1.48	11.88	0.74	26.35	5.02	816.65	48.01
Gallic acid	6	35.55	0.21	29.40	2.62	24.20	1.27	1327.50[b]	142.13
TBHQ	6	21.98	2.93	35.30	0.49	9.92	1.53	300.75[b]	116.46
Blank		4.69	0.35	11.33	0.19	34.93	2.69	208.60	46.39
								860.45[c]	292.11

[a]Heating time for FAME conc. of 6 μmol/5 g = 17.5 h, FAME conc. 12 μmol/5 g = 18 h, oil conc. of 6 μmol/5 g = 20 h, and oil conc. of 12 μmol/5 g = 25 h. FAME heating was at 90°C, and oil heating was at 100°C due to the oils' high oxidative stability index (OSI) values.
[b]Heating time was 48 h.
[c]Heating time was 25 h, as for the 12 μmol/5 g concentration treatment for oil.

TABLE 2
Summary of Statistical Analysis of Various Compounds as Antioxidants in FAME and Soybean Oil Systems[a]

	OSI				Viscosity			
	FAME		Oil		FAME		Oil	
	P value at 5%	LSD value	*P* value at 5%	LSD value	*P* value at 5%	LSD value	*P* value at 5%	LSD value
Compounds	<0.0001	1.445	<0.0001	1.199	<0.0001	4.72	<0.0001	151.8
Concentration	0.0750	0.588	0.3470	0.490	0.0060	1.93	<0.0001	62.0
Replication	0.0570	0.588	0.0090	0.490	0.0430	1.93	<0.0001	62.0
Cpds × concentration	<0.0001		0.8940		0.3270		<0.0001	

[a]LSD, least significant difference; Cpds, compounds; for other abbreviation see Table 1.

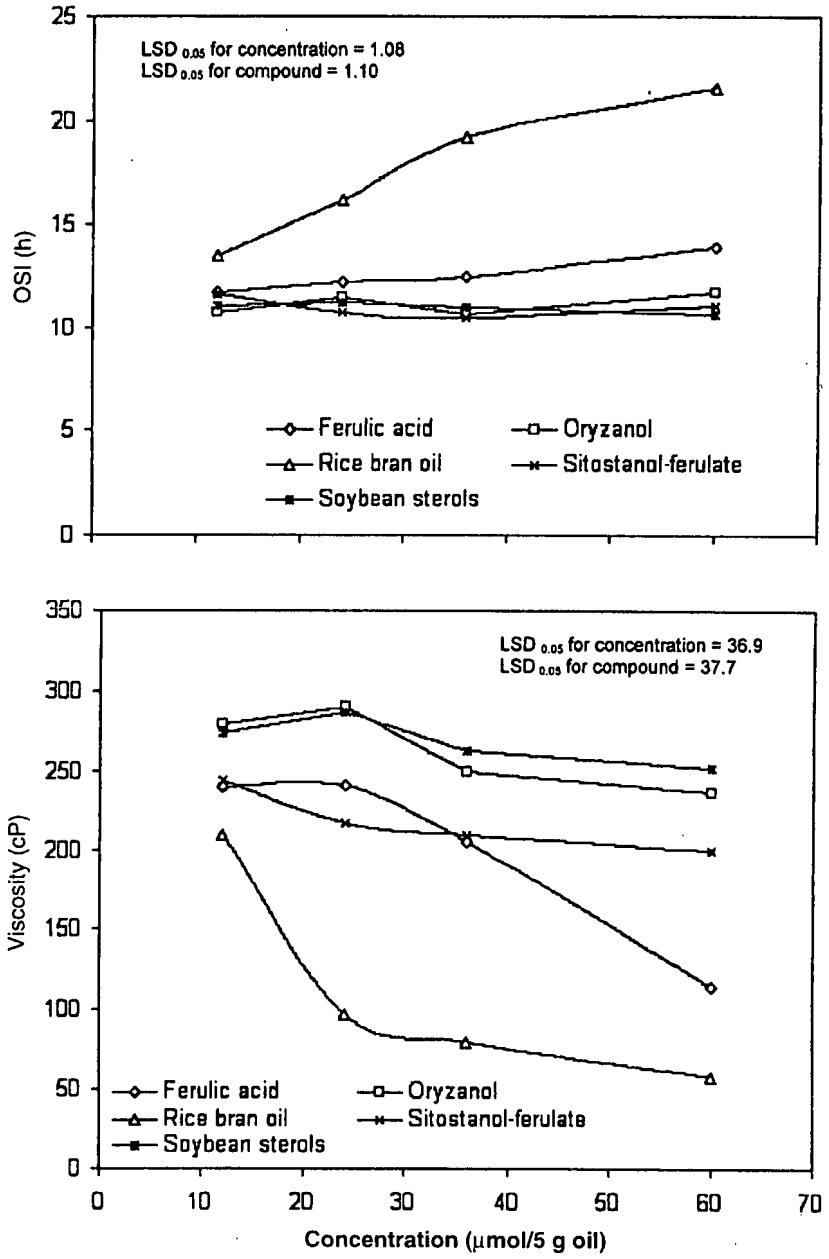

FIG. 1. Antioxidant activity of selected agents at various concentrations in soybean oils. LSD, least significant difference.

The viscosity developed in oil was much higher than for FAME because of its TAG structure, compared with the single acyl chain in FAME. We also noticed that the higher-concentration samples had much higher viscosity than the lower-concentration samples, owing to the difference in heating time. The high-concentration oil was heated for 25 h, the low-concentration oils for 20 h. Therefore, the expected viscosity reduction at the higher concentration was not seen. This observation indicated that once the oil is oxidized, the polymerization reaction can proceed very rapidly. TBHQ again showed its significant anti-polymerization effect in oil compared with gallic acid.

Antioxidant activity of selected compounds at various concentration levels. The effect of type and concentration of selected compounds is shown in Figure 1. The interaction between type and concentration was significant for OSI but not for viscosity at the 5% probability level. Therefore, the main effects of type and concentration on viscosity were examined (Fig. 2).

The oxidative stability of oil was significantly affected by type of compounds tested and their concentration. Rice bran oil had significantly better antioxidant activity than the others, and its activity increased markedly with its concentration

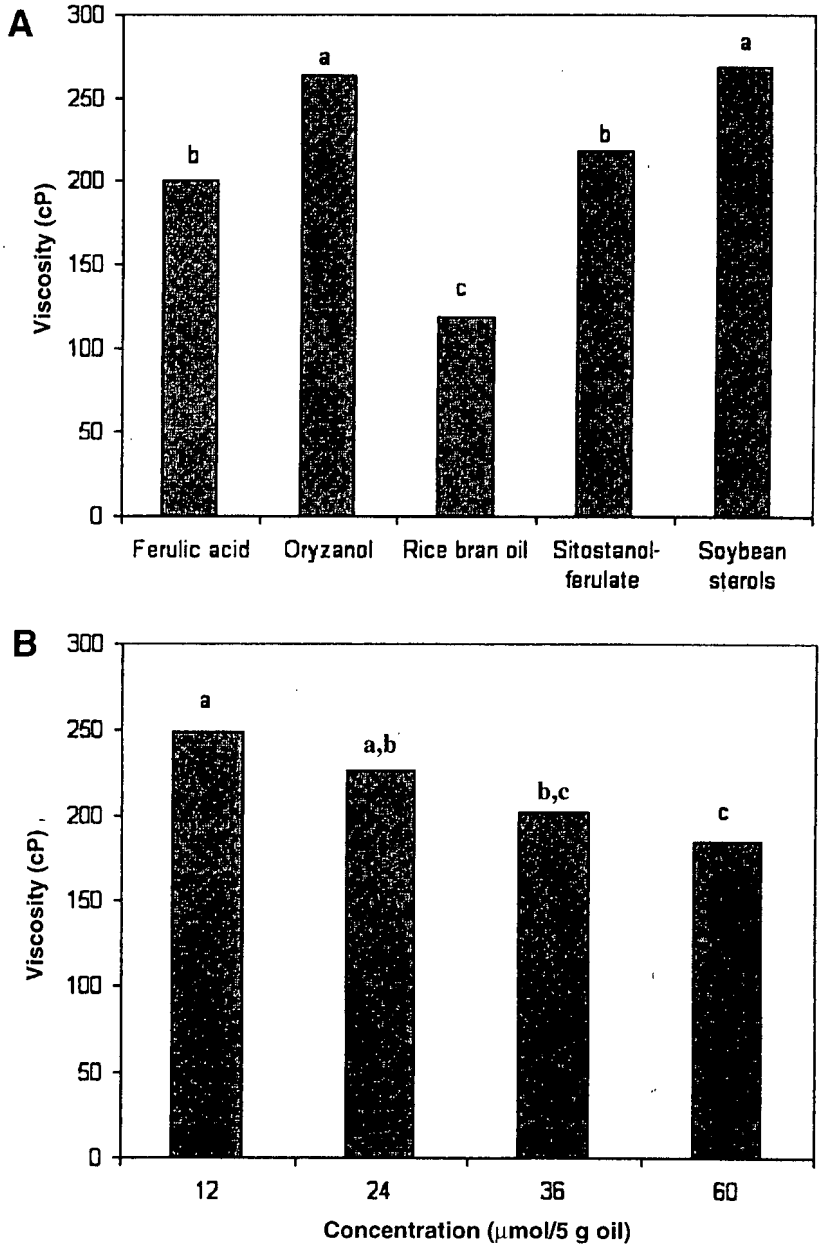

FIG. 2. Main effects of antioxidant type and concentration on viscosity of oxidized soybean oil. Note: Different letters at top of bars indicate statistical difference at 5% probability level.

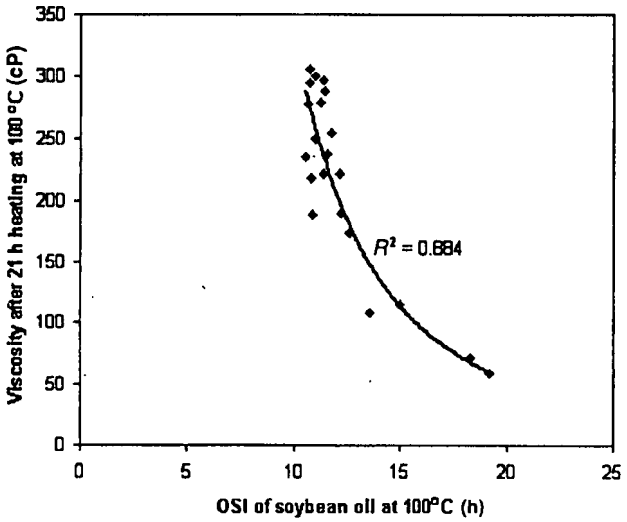

FIG. 3. Relationship of oxidative stability and polymerization of soybean oil. OSI, oxidative stability index.

in an oil. Ferulic acid had similar activity compared with others at lower concentration but showed improved activity at higher concentration.

Viscosity of the oil was also significantly affected by type and concentration of the compounds. Rice bran oil was the most effective agent in preventing polymerization, and its activity increased dramatically with an initial increase in concentration but tended to level off at higher concentration. The effectiveness of ferulic acid as an antipolymerization agent was more evident at higher concentration than at lower concentration. Sitostanyl-ferulate showed significantly more antipolymerization activity than did oryzanol and soybean sterols. It is not clear why sitostanyl ferulate was more effective at the concentration tested than oryzanol, which is a mixture of ferulic acid esters of various triterpene alcohols that are structurally very similar to phytosterols, including cycloartenol, 24-methylene-cycloartenol, and a small amount of campesterol. These observations also suggest that the good antioxidant/antipolymerization activity of rice bran oil may not be due to its oryzanol content alone but to other minor lipid components, such as avenasterols. The phytosterol avenasterol, constituting 32% of the total phytosterols in oats), greatly reduced the degree of deterioration of soybean oil at frying temperature (180°C) (4,5). The content of tocopherol and tocotrienol in rice bran oil is usually relatively low, about 20 times less than its phytosterol content; therefore, its contribution to the oxidative stability of the system may be very small. The quantity of rice bran oil added in the concentration levels in this experiment ranged from 0.17 to 1.7 g in 5 g of oil substrate. Other minor components, in addition to avenasterol, also may have contributed to the antioxidant activity of rice bran oil.

The antioxidant activity of phytosterols also was studied by other researchers. Xu and Godber (18) examined the antioxidant activity of the major components of rice bran oryzanol in a linoleic acid model system, at slightly elevated temperature and by monitoring peroxide formation. They found that the antioxidant activities of various steryl ferulates were lower than those with ferulic acid and α-tocopherol. The concentration levels used in their study (1:100 to 1:500, sterol/substrate, molar ratio) were similar to those used in our study (approximately 1:100 to 1:1000 for oil substrate, and 1:300 to 1:3000 for FAME substrate). At a 1:500 ratio of sterol to substrate, Xu and Godber (18) detected no antioxidant activity. The antioxidant activity of oryzanol also was reported as not significant at concentrations lower than 0.5% (1:300 molar ratio of sterol to oil substrate) (19). If we had used a higher concentration of phytosterols in our study, we might have observed higher antioxidant/antipolymerization activity of the steryl ferulate.

There has been some concern expressed about the high-temperature testing of oxidative stability of oil and antioxidant activity because of the rapid oxidation, polymerization, and decomposition of antioxidant (20). Nevertheless, there is also evidence that the OSI method gives a reliable estimation of oil stability, and it is an AOCS recommended standard method. The OSI method should be particularly useful in evaluating stability of frying oil and the effectiveness of antioxidants used in this type of oil.

Various lipid materials could be used as substrates to test the activities of antioxidants. Methyl esters of vegetable oils should be an ideal system to evaluate antioxidant activity, because they can be purified easily to ensure that no other minor lipid components interfere with the evaluation. Methyl linoleate was successfully used as a model system for antioxidant activity study by OSI (21). The only drawback with soybean FAME was that it did not develop significant viscosity. Therefore, oil may be a better substrate to evaluate the antipolymerization effect of antioxidants.

The relationship of oxidative stability and viscosity development is shown in Figure 3. Oils with similar stabilities as indicated by the OSI value may have quite different viscosities. This observation indicates that different antioxidants may work differently. Some may prevent peroxide formation. Therefore, the subsequent reactions are delayed. Others may not prevent peroxide formation, but they may delay the subsequent polymerization reaction.

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Antioxidant Effects of Phytosterol and Its Components

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Summary Phytosterol contained in vegetable oils is known to exert a hypocholesterolemic function. In the present study, the antioxidant effects of phytosterol and its components, β-sitosterol, stigmasterol, and campesterol, against lipid peroxidation were examined by making a comparison with 2,2,5,7,8-pentamethyl-6-chromanol (PMC). It was found that these compounds exerted antioxidant effects on the oxidation of methyl linoleate in solution and its effect decreased in the order of: PMC≫phytosterol∼campesterol∼β-sitosterol>stigmasterol. Phytosterol also suppressed the oxidation and consumption of α-tocopherol in β-linoleoyl-γ-palmitoyl phosphatidylcholine (PLPC) liposomal membranes, the effects being more significant than dimyristoyl PC of the same concentration. Stigmasterol accelerated the oxidation of both methyl linoleate in solution and PLPC liposomal membranes in aqueous dispersions, which was ascribed to the oxidation of allylic hydrogens at the 21- and 24-positions. Taken together, the present study shows that phytosterol chemically acts as an antioxidant, a modest radical scavenger, and physically as a stabilizer in the membranes.

Key Words antioxidant, free radical, lipid peroxidation, phytosterol

The oxidation of biological molecules such as lipids, proteins, and DNA by molecular oxygen induced through active oxygen and nitrogen species is accepted to be involved in the development of numerous disorders and pathological events such as atherosclerosis, cancer, and even aging processes (*1*, *2*). Such oxidation usually proceeds by a free radical-mediated chain mechanism, and the chain-breaking antioxidants such as vitamin E suppress the oxidation and protect biological molecules and tissues from oxidative damage (*3–6*). Plant sterols, also called phytosterols, are reported to include over 250 different sterols (*7*), the most common representatives being β-sitosterol, stigmasterol, and campesterol (structures are shown in Fig. 1). Although they have received much attention because of their hypocholesterolemic property (*8–11*), there are few reports available regarding the antioxidant effects of phytosterol components. Some investigators have studied phytochemicals, mixture sterols (*12–15*), and attributed their antioxidant activities to the formation of an allylic free radical and its isomerization to other relatively stable free radicals (*15*). Wang et al. (*16*) examined the effect of phytosterols on the stability of oils against oxidation by heating.

The present study has been carried out to clarify the reactivities of sterols, especially phytosterol components, toward the peroxyl radicals and their actions as antioxidants in several model systems.

MATERIALS AND METHODS

Chemicals. Commercial β-linoleoyl-γ-palmitoyl phosphatidylcholine (PLPC) and dimyristoyl phosphatidylcholine (14:0 PC) were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and used without further purification. The liposomes were prepared as follows. PLPC and lipid-soluble additives, when required, were dissolved in methanol and the solution was placed into a pear-shaped flask. Methanol was removed by evacuation on a water aspirator using a rotary vacuum evaporator to obtain a thin film on the flask wall. An appropriate amount of phosphate-buffered saline (PBS, pH 7.4) was added and the film was slowly peeled off by

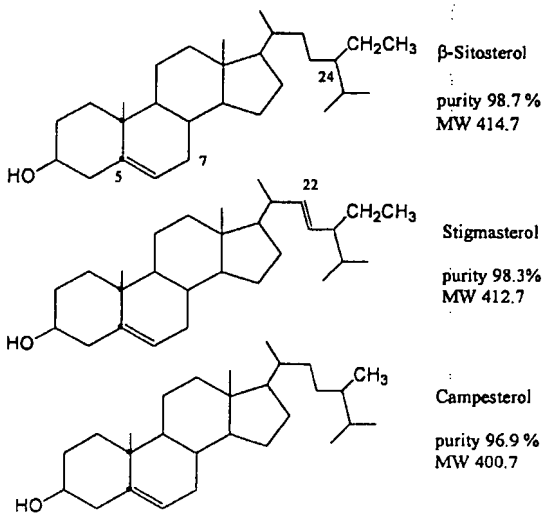

Fig. 1. Chemical structures of phytosterols used in this study. Molecular weight and purity are also shown in the figure.

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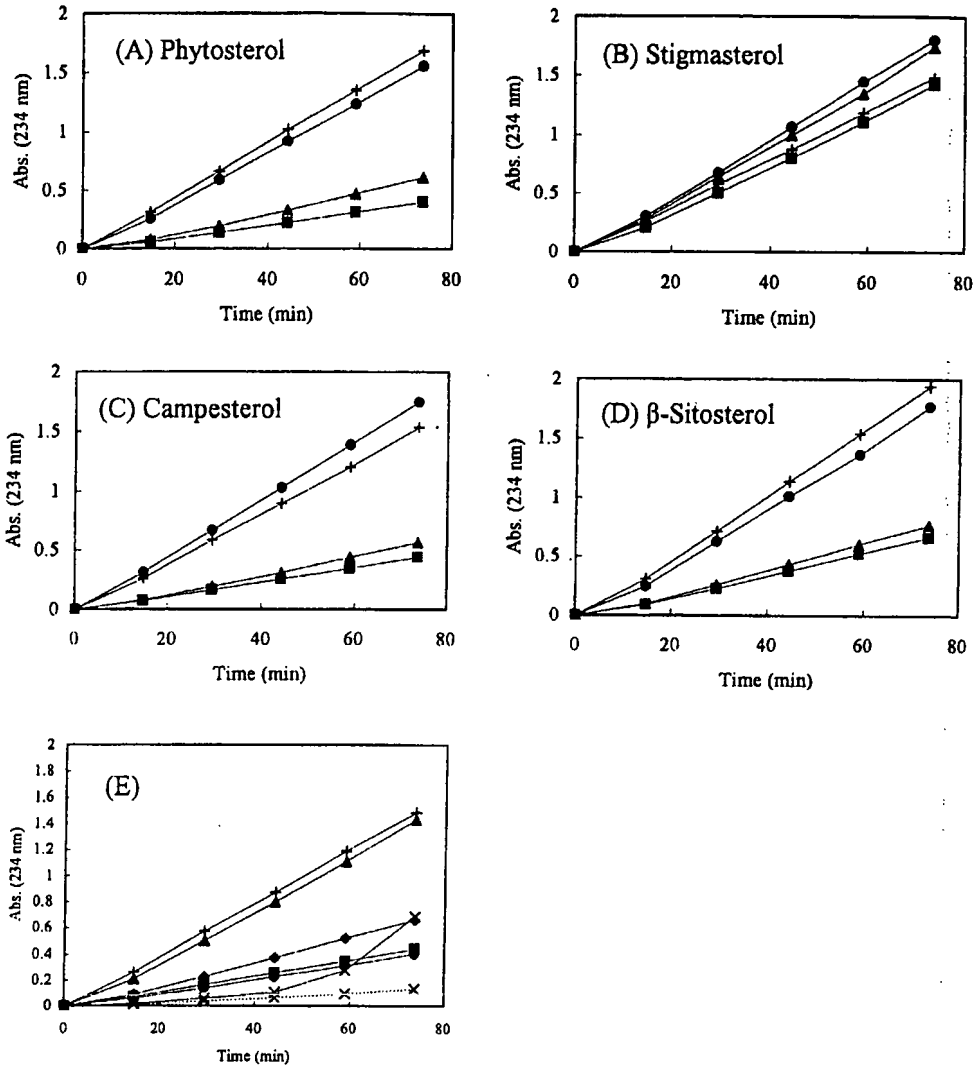

Fig. 2. Antioxidant effects of phytosterols and PMC in solution. The increase in absorption at 234 nm was followed in the oxidation of methyl linoleate (7.4 mM) induced by AMVN (1.0 mM) in acetonitrile at 37°C in the absence (plus) and presence of either phytosterol (A), stigmasterol (B), campesterol (C), and β-sitosterol (D) (circle: 10, triangle: 50, square: 100 μM). In the figure (E), the antioxidant effects of phytosterol (circle), stigmasterol (triangle), campesterol (square), and β-sitosterol (diamond) (100 μM) were compared with PMC (cross and solid line, 5 μM; cross and broken line, 10 μM) under the same experimental conditions.

shaking to obtain white, milky liposome suspensions. Methyl linoleate (MeLH) was obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan) and used as received. Natural phytosterol, β-sitosterol, stigmasterol, and campesterol were kindly supplied from Tama Biochemical Co. Ltd. (Kanagawa, Japan). The phytosterol used contained 48.8 wt% β-sitosterol, 14.2 wt% stigmasterol, and 29.6 wt% campesterol and included 7.4 wt% of other compounds. The purities of β-sitosterol, stigmasterol, and campesterol used were 98.7, 98.3, and 96.9 wt% (determined by GLC), respectively. Natural D-α-tocopherol and 2,2,5,7,8-pentamethyl-6-chromanol (PMC) were kindly supplied from Eisai Co. Ltd. (Tokyo, Japan). 2,2'-Azobis(2,4-dimethylvaleronitrile) (AMVN), used as a lipophilic radical initiator, was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Oxidation of MeLH and PLPC. The oxidation of MeLH and PLPC induced by radical initiators was performed in acetonitrile solution and aqueous dispersions, respectively, at 37°C in air. The accumulations of PLPC hydroperoxides (PLPCOOH) and MeLH hydroperoxides (MeLOOH) were followed by HPLC using an UV detector at 234 nm and a spectrophotometer (Shimadzu UV-2450) with absorption at 234 nm, respectively. A silica-gel column (5 μm, 250×4.6 mm, Wako) was used and methanol/40 mM phosphate buffer (9/1 by volume) was delivered as the eluent at 1 mL/min for the PLPCOOH analysis system.

Reproducibility. The experiments reported in this paper were repeated, in general, several times and the results were reproducible within the experimental error of ±5%.

RESULTS AND DISCUSSION

Inhibition of oxidation of methyl linoleate by phytosterols

The oxidation of linoleic acid and its esters initiated by free radicals is known to proceed by a free radical chain mechanism to give four conjugated diene hydroperoxides quantitatively (*17*), and hence this is a convenient and reliable system to evaluate the antioxidant activity of radical-scavenging antioxidants (*18*). The activities of phytosterol and its major components, β-sitosterol, stigmasterol, and campesterol, as antioxidants were examined in the oxidation of methyl linoleate in an acetonitrile solution. The initiating radicals were formed by the decomposition of a lipid-soluble radical initiator, AMVN, in the presence of oxygen. The extent of oxidation was measured from strong absorption at 234 nm due to conjugated diene. The effect of PMC, a vitamin E homologue, was also measured for comparison. Phytosterol, β-sitosterol, stigmasterol, and campesterol exerted an antioxidant effect against the oxidation of methyl linoleate induced by peroxyl radicals in a concentration-dependent manner (Fig. 2A–D), the effect decreasing in the order of PMC≫phytosterol ~ campesterol~β-sitosterol>stigmasterol. Interestingly, phytosterol, a mixture of phytochemicals, showed the most effective antioxidant activity among the sterols studied. The reason is not clear; however, the other components (7.4 wt%) may possess high antioxidative properties. PMC exerted a much higher antioxidant effect than any other sterol, implying that the hydroxyl group of the chroman ring is much more reactive toward peroxyl radicals than the hydroxyl group and allylic hydrogens of the cholesterol ring.

Action of phytosterols in PLPC liposomal membranes

The actions of phytosterol, β-sitosterol, stigmasterol, and campesterol as antioxidants were then studied in liposomal membranes prepared from phosphatidylcholine (PC). As shown in Fig. 3, no clear antioxidant effect was observed for any of the sterols (100 μM) under this experimental condition. Moreover, stigmasterol exerted a prooxidant effect. The reason for the prooxidant effect of stigmasterol is not clear at present, but considering the structural difference (Fig. 1), the prooxidant action of stigmasterol may be ascribed to the allylic hydrogens at positions 21 and 24, which may take part in the chain reaction in the PLPC liposomal membranes.

Phytosterol functions as a structural component in plant membranes. The effect of phytosterol at higher concentrations was then studied regarding membrane oxidation. It was found that 26 mol% of phytosterol (1.00 mM against 2.83 mM PLPC) exerted a significant effect on the oxidation of PLPC membranes (Fig. 4). Interestingly, 14 : 0 PC of the same concentration exerted a much less significant effect, showing that the significant antioxidant effect of phytosterol cannot be attributed solely to lowering the concentration of oxidizable lipids, that is, bisallylic hydrogens.

The effect of α-tocopherol against the oxidation of PLPC liposomal membrane was studied in the absence and presence of 14 : 0 PC or phytosterol. The results are also included in Fig. 4. α-Tocopherol acts as a less efficient antioxidant in the membranes than in homogeneous solution, but it suppressed the oxidation of PLPC in the absence and presence of 14 : 0 PC in the membrane similarly. On the other hand, the effect of phytosterol overwhelmed that of α-tocopherol and the apparent effect of α-tocopherol was small (Fig. 4). The rates of consumption of α-tocopherol under these con-

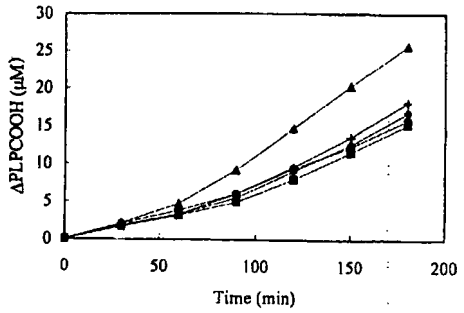

Fig. 3. Oxidation of PLPC liposome (2.83 mM) induced by 1.0 mM AMVN in the absence (plus) and presence of either 100 μM phytosterol (circle), stigmasterol (triangle), campesterol (square), or β-sitosterol (diamond) at 37°C. Formation of PLPC hydroperoxides was conducted as described in Materials and Methods.

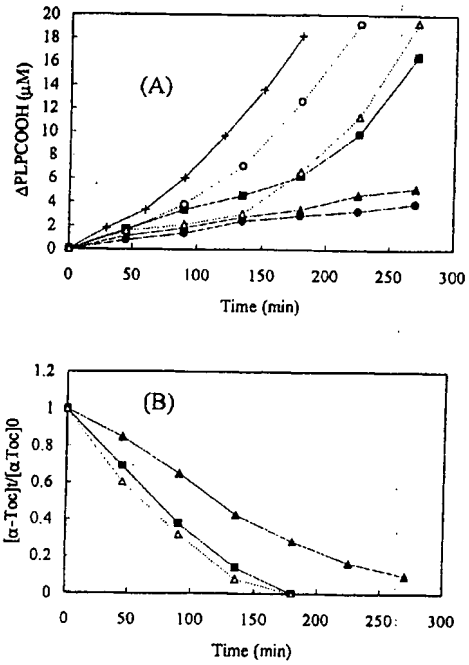

Fig. 4. Oxidation of PLPC liposome (2.83 mM) induced by 1.0 mM AMVN in the absence (plus) and presence of either 1 mM phytosterol (solid circle), 1 mM 14 : 0 PC (open circle), 2 μM α-tocopherol (solid square), 1 mM phytosterol and 2 μM α-tocopherol (solid triangle), or 1 mM 14 : 0 PC and 2 μM α-tocopherol (open triangle) at 37°C. 14 : 0 PC and α-tocopherol were incorporated into PLPC liposomal membranes simultaneously. Formation of PLPC hydroperoxides (A) and consumption of α-tocopherol (B) were conducted as described in Materials and Methods.

ditions are shown in Fig. 4B. There was little effect with 14 : 0 PC, while phytosterol delayed the consumption of α-tocopherol.

In conclusion, the present study shows that phytosterol chemically acts as an antioxidant, a modest radical scavenger, in solution and physically in the membranes by packing and stabilizing the membrane. These results imply the possible role of phytosterols in vivo, which is another interesting future subject.

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